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Brain-Plasma Distribution of Free and Total Benzodiazepines in Dogs Physically Dependent on Different Doses of Diazepam

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WALA, E. P., W. R. MARTIN AND J. W. SLOAN. *Brain-plasma distribution of benzodiazepines in dogs physically dependent on different doses of diazepam.* PHARMACOL BIOCHEM BEHAV **52**(4) 707-713, 1995. – Steady-state levels of oxazepam (OX), nordiazepam (ND), and diazepam (DZ) in plasma, brain tissue, cerebrospinal fluid (CSF), and intracranial microdialysis perfusate were determined in dogs dependent on 0.56, 4.5, 9, and 36 mg/kg per day of DZ. There was a linear relationship between the total plasma and brain levels of DZ, ND, and OX and the chronic dose of DZ. Levels of free benzodiazepines in plasma and CSF and levels in microdialysis perfusates from plasma and brain were significantly correlated. With increasing dependence on DZ there was progressively more free ND and OX and less free DZ in plasma, CSF, and brain. There was a correlation between several signs of precipitated abstinence and free ND in the brain interstitial fluid, whereas convulsions emerged only when free metabolites exceeded free DZ. The changes in contribution of free DZ, ND, and OX to the overall levels of benzodiazepines present in the CNS may explain differences in signs of abstinence for different levels of dependence on DZ.

Intracerebral microdialysis Brain-plasma benzodiazepine distribution Diazepam Nordiazepam Oxazepam Physical dependence on diazepam

MULTIPLE-DOSE administration of diazepam (DZ) results in an accumulation of DZ and/or its active metabolites, nordiazepam (ND) and oxazepam (OX), in humans (16), dogs (26,30,31,42), and rats (29). There are several lines of evidence suggesting that the accumulation of total benzodiazepines in plasma and in brain tissue (28) and high levels of free benzodiazepines in the extraneuronal brain space (29,41) have an important role in the induction of physical dependence. As previously reported, dependence on DZ is very complex and probably involves multiple modes of action and interaction between DZ and its metabolites, ND and OX (28). Furthermore, DZ and ND produce different types of physical dependence as indicated by the time courses and individual signs of abstinence observed during withdrawal (30) and precipitation (31). In dogs (37), baboons (27), and rats (43), physical dependence on DZ, as indicated by precipitated abstinence scores, increases with the chronic dose of DZ; however, the individual signs of abstinence increase, decrease, or tend to be U-shaped. Thus, it cannot be ruled out that the differences in individual signs of abstinence observed for the different levels of dependence on DZ are not due to altered pharmacokinetics and to changes in the mutual relationship between levels of free DZ and its metabolites, ND and OX, within the CNS.

The good correlations between the free fraction of benzodiazepines in plasma and the levels in cerebrospinal fluid (4), and between the high-performance liquid chromatography (HPLC) retention time and brain uptake (5) as well as the brain-plasma distribution in dogs stabilized on DZ or ND (41) and in rats infused with DZ (11) indicate that protein-unbound (free) benzodiazepines are transported across the blood-brain barrier and that the free plasma levels approximate the free levels in the brain. Previously reported data on plasma and brain levels of benzodiazepines were determined either in acute experiments or after stabilization on a single dose of benzodiazepine; the brain-plasma distribution of DZ and its metabolites has not been evaluated in subjects dependent on different doses of DZ.

Thus, we undertook the present study to assess the steady-

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state levels of free and total DZ and its active metabolites, ND and OX, in plasma, brain tissue, cerebrospinal fluid (CSF), and the extraneuronal brain space (intracranial microdialysis) in dogs chronically administered a wide range of doses of DZ. The generated results provided an opportunity to compare the relationship between interstitial unbound DZ and its metabolites at different levels of physical dependence on DZ, and to correlate the individual signs of precipitated abstinence with free levels of benzodiazepines at the receptor site(s).

METHODS

Animals

We collected data from female beagle-type dogs (body weight about 10 kg) that were used to study physical dependence on DZ (37). Briefly, the dogs were stabilized for 77–110 days on 0.56, 4.5, 9, and 36 mg/kg per day (three times per day) of orally administered DZ and were precipitated weekly with graded doses of oral flumazenil (0.66–72 mg/kg) and placebo (modified Latin square design) and observed for signs of abstinence as previously described (37). The scores for each sign of abstinence evoked by the administration of 18 mg/kg flumazenil are presented here as the mean areas under time action curves with the lactose placebo subtracted.

Microdialysis of Brain and Plasma

The dogs were subjected to intracranial microdialysis approximately a week after the last precipitation. One hour after the morning dose of DZ the dogs were anesthetized with pentobarbital (30 mg/kg, IV) and placed in a stereotaxic instrument. The CMA/10 3-mm microdialysis probes (Carnegie Medicin BAS, West Lafayette, IN) were implanted bilaterally 3 mm deep into the parietal cortex as described previously (41). Before and during the implantation procedure the probes were perfused with artificial cerebrospinal fluid (ACSF) (126.5 mM NaCl, 27.5 mM $NaHCO_3$, 2.4 mM KCl, 0.5 mM KH_2PO_4, 1.1 mM $CaCl_2$, 0.85 mM $MgCl_2$, 0.5 mM $NaSO_4$, glucose 5.9 mM, adjusted to pH 7.5) at a rate of 10 μl/min. Then, 5 min after implantation of the probes, the flow rate was reduced to 2 μl/min and was maintained at this level throughout the experiment. After a 25-min equilibration period, the three samples of the brain perfusates were collected at 25-min intervals. At the end of the experiment the probes were washed by perfusing for 30 min with distilled water at a rate of 10 μl/min and afterward inserted into plasma and perfused at room temperature with an isotonic phosphate buffer (2 μl/min). After 25 min of stabilization, two 25-μl samples of the plasma perfusates were collected at 25-min time intervals. Although the experiment lasted for about 3–4 h, we have previously shown that levels of DZ and its metabolites are stable during 8-h dosing intervals (41).

Brain tissue, blood, and CSF were collected at the end of microdialysis (about 4 h after the last dose of DZ) as previously described (41).

Calibration of the Probes

In vitro calibration (40) was performed as previously described (41). Briefly, the tips of the probes were inserted into the mixtures of OX, ND, and DZ (0.2–10 μg/ml in ACSF) and perfused with ACSF at a rate of 2 μl/min. The concentrations of the drugs in collected perfusates and in the outer medium were determined and the relative recoveries were calculated for each drug and probe used. Furthermore, each probe was calibrated at the end of the microdialysis experiment by immersing it in a 1-μg/ml mixture of benzodiazepines and perfusing with ACSF, as described earlier. The mean (n = 32) recoveries were equal to 27.8 ± 2.0%; 19.0 ± 1.6%; and 15.9 ± 2.03% for OX, ND, and DZ, respectively. Because of the experimental design, the recovery of DZ and its metabolites from microdialysis probes was not determined in vivo. Because the in vitro calibration employed tends to underestimate levels in the surrounding tissues (7), the data are presented as relative concentrations in brain and plasma perfusates.

Analysis of Drugs

The concentrations of benzodiazepines in microdialysis perfusates, brain tissue, CSF, and plasma were determined by HPLC as described previously (29,37,41). Briefly, the 50-μl samples of microdialysis perfusates or CSF were spiked with 10 μl of internal standard (10 μg/ml methanol solution of flunitrazepam) and then 50 μl was injected into the HPLC column. The levels of total benzodiazepines in plasma or brain tissue (parietal cortex) were determined after solid-phase extraction of plasma and brain homogenates on Bond Elut C18 (1 cc/100 mg and 3 cc/100 mg) columns (Infolab, Clarksville, MI).

Plasma Protein Binding

For each dog, the extent of binding of OX, ND, and DZ to plasma protein was determined in plasma samples collected at the last week of the chronic studies. Plasma samples (0.8 ml) were dialyzed against isotonic phosphate buffer at 37°C for 20 h and concentrations of benzodiazepines were determined in dialysates and plasma by HPLC as previously reported (41). Free fractions were calculated as the ratio of free (dialysate) and total (postdialysis plasma) concentrations. The free steady-state plasma levels of benzodiazepines were estimated as the product of the total levels multiplied by the free fractions.

Statistics

Statistical methods included linear regression analysis and one-way analysis of variance (ANOVA).

RESULTS

The present data indicate that in dogs dependent on different doses of DZ, steady-state levels of ND and OX in plasma, brain tissue (parietal cortex), and CSF had a statistically significant positive regression with the chronic dose of DZ (Fig. 1A). Although the total levels of DZ in plasma and brain tissue also significantly increased with dose, the levels of free DZ in CSF did not increase. It is noteworthy that levels of ND increased proportionally to the chronic dose of DZ, whereas the levels of DZ increased less than was predicted from the dose. When the dose of DZ increased from 0.56 to 36 mg/kg per day (64 times), the total plasma levels of DZ increased only threefold (from 0.36 to 1.3 μg/ml), levels of OX change about 15-fold (from 0.21 to 2.99 μg/ml), and the levels of ND about 50-fold (from 0.24 to 11.4 μg/ml), respectively. Thus, the plasma concentration of DZ decreased from about 45% of the total benzodiazepines (DZ + ND + OX) concentration to 8% for doses of 0.56 and 36 mg/kg per day, respectively.

There was a highly significant correlation between levels of total DZ, ND, and OX in plasma and brain tissue (parietal cortex) (Fig. 1B). No evidence was found for the higher accumulation of benzodiazepines in brain than plasma. At steady-

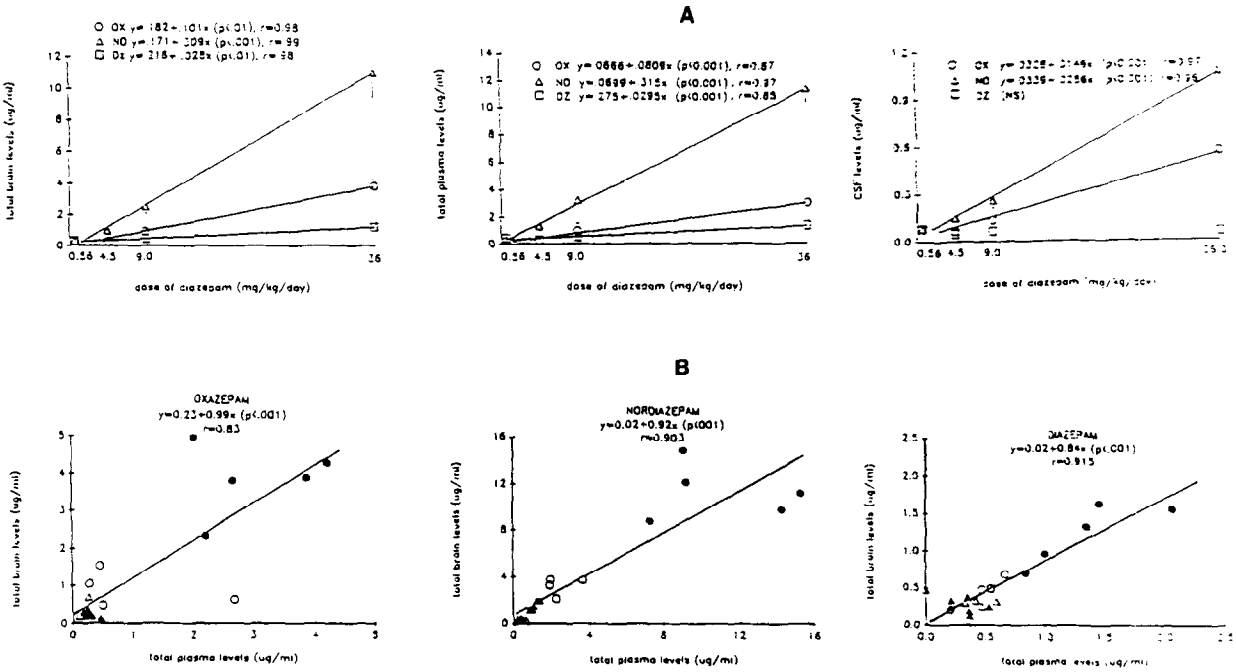

FIG. 1. (A) Relationships between steady-state levels of diazepam (DZ), nordiazepam (ND), and oxazepam (OX) in plasma, brain tissue, and cerebrospinal fluid (CSF) and the stabilization dose of DZ. The values are the mean ± SEM of six (0.56 and 4.5 mg/kg per day), four (9 mg/kg per day), and five (36 mg/kg per day) dogs. (B) Distribution of total OX, ND, and DZ between plasma and the brain tissue (parietal cortex) in dogs physically dependent on 0.56 (△ △), 4.5 (▲ ▲), 9 (○ ○), and 36 (● ●) mg/kg per day of DZ. The points represent the individual dogs.

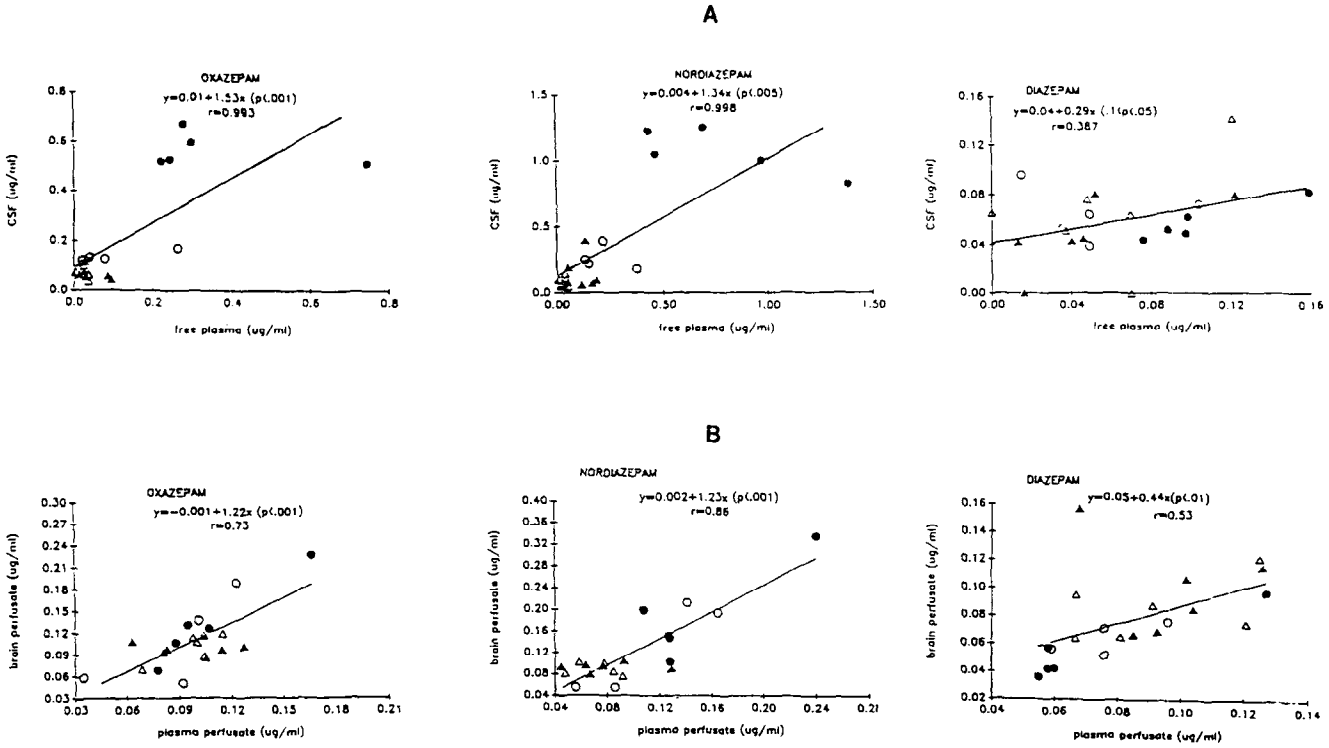

FIG. 2. Distribution of free oxazepam (OX), nordiazepam (ND), and diazepam (DZ) between plasma (estimated from total plasma levels and free fractions) and cerebrospinal fluid (CSF) (A) and between plasma and brain microdialysis perfusates (relative concentrations) (B). Dogs were physically dependent on 0.56 (△ △), 4.5 (▲ ▲), 9 (○ ○), and 36 (● ●) mg/kg per day of DZ. The points represent the individual dogs.

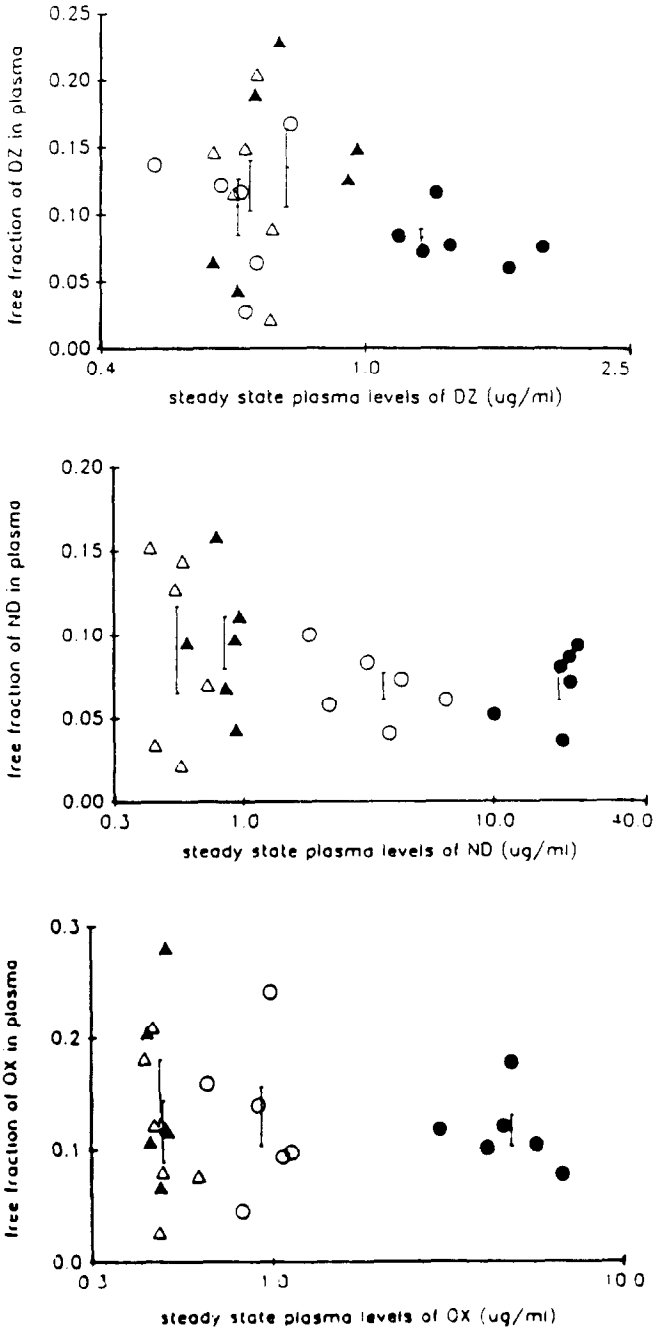

FIG. 3. Free fractions of diazepam (DZ), nordiazepam (ND), and oxazepam (OX) in relation to steady-state total plasma levels in dogs chronically dosed with 0.56 (△ △), 4.5 (▲ ▲), 9 (○ ○), and 36 (● ●) mg/kg per day of DZ. The extent of plasma protein binding was determined in the last week of dependence study (week 10 or 12 of stabilization). The individual and mean (± SEM) values are presented for each group of dogs. Data also include two dogs (9 mg/kg per day) and one dog (36 mg/kg per day) that died before or during the microdialysis experiment.

state the brain-plasma concentration ratios were equal to about 1 and did not change with the chronic dose of DZ.

There was a significant correlation between free levels of OX and ND in CSF and plasma (levels estimated from total plasma levels and free fractions) (Fig. 2A). Although free plasma-CSF ratios for ND and OX were approximately equal to 1, there was variation between dogs receiving the same stabilization dose. A poor correlation was found between free levels of DZ in CSF and plasma.

Levels of free ND and OX in microdialysis perfusates from brain and plasma were significantly correlated (Fig. 2B). The concentrations ratios in brain-plasma perfusates were equal to about 1; however, the free levels in perfusates varied markedly between dogs either within or between stabilization doses. Once again, there was a poor correlation between levels of free DZ in perfusates from brain and plasma perfusates when data collected from all dogs were analyzed together. It is noteworthy, however, that brain-plasma perfusate concentration ratios for DZ approximated 1 (1.1, 1, 1.3, and 1.3) when they were calculated for dogs stabilized with 0.56, 4.5, 9, and 36 mg/kg per day of DZ, respectively.

Figure 3 indicates that there was not a significant regression (df = 1,22) of free fractions on total steady-state plasma levels for OX (F = 0.66), ND (F = 0.013), or DZ (F = 2.5). Furthermore, as indicated by one-way ANOVA (between doses, df = 3,20) plasma protein binding of OX (F = 0.354), ND (F = 2.46), and DZ (F = 2.27) did not change with the dose of DZ. As can be seen, however, the extent of binding showed high variability between dogs dependent on the same dose of DZ.

Figure 4A indicates that in dogs stabilized with low doses of DZ (0.56 and 4.5 mg/kg per day), the free levels of OX, ND, and DZ in the intracranial perfusate were about equal (OX/DZ and ND/DZ = 1), whereas in dogs dependent on the higher doses of DZ (9 and 36 mg/kg per day), there was progressively more free ND and OX and less free DZ. An attempt to correlate the individual signs of precipitated abstinence with the free levels of benzodiazepines in the brain revealed that in DZ-dependent dogs, myoclonus and clonic convulsions were flumazenil-evocable only when the free levels of metabolites were higher than the levels of DZ (OX/DZ and ND/DZ > 1) (Fig. 4B and C).

Figure 5(A-E) illustrates that scores for several other signs of abstinence (limb, neck, and whole-body tremors, twitches and jerks, rigid walking, and lip licking) correlated well with free levels of ND in the extraneuronal brain space (intracranial perfusate) at each level of dependency. Signs of abstinence and free levels of OX (which parallel free levels of ND) were also well correlated (data not shown). No relationship was found between individual signs of precipitated abstinence and levels of free DZ.

DISCUSSION

The present results indicate that in dogs physically dependent on 0.56-36 mg/kg per day of DZ, the free levels of ND and OX were approximately equal in plasma and the brain perfusates, and likewise the free levels of these metabolites in plasma approximated their levels in CSF. These observations support previous reports in which similar free plasma and CSF levels of several acutely administered benzodiazepines were found (4,17,19,23) and in which DZ was about equally distributed between plasma and the extraneuronal brain space in rats (11) and dogs (41) stabilized with a single dose of DZ. The present data indicate, however, that among dogs there is high variability in the distribution of benzodiazepines between brain-plasma perfusates and CSF-plasma, which is also the case with CSF-plasma data reported previously (4,6). Thus, it must be emphasized that in the individual, the accurate predic-

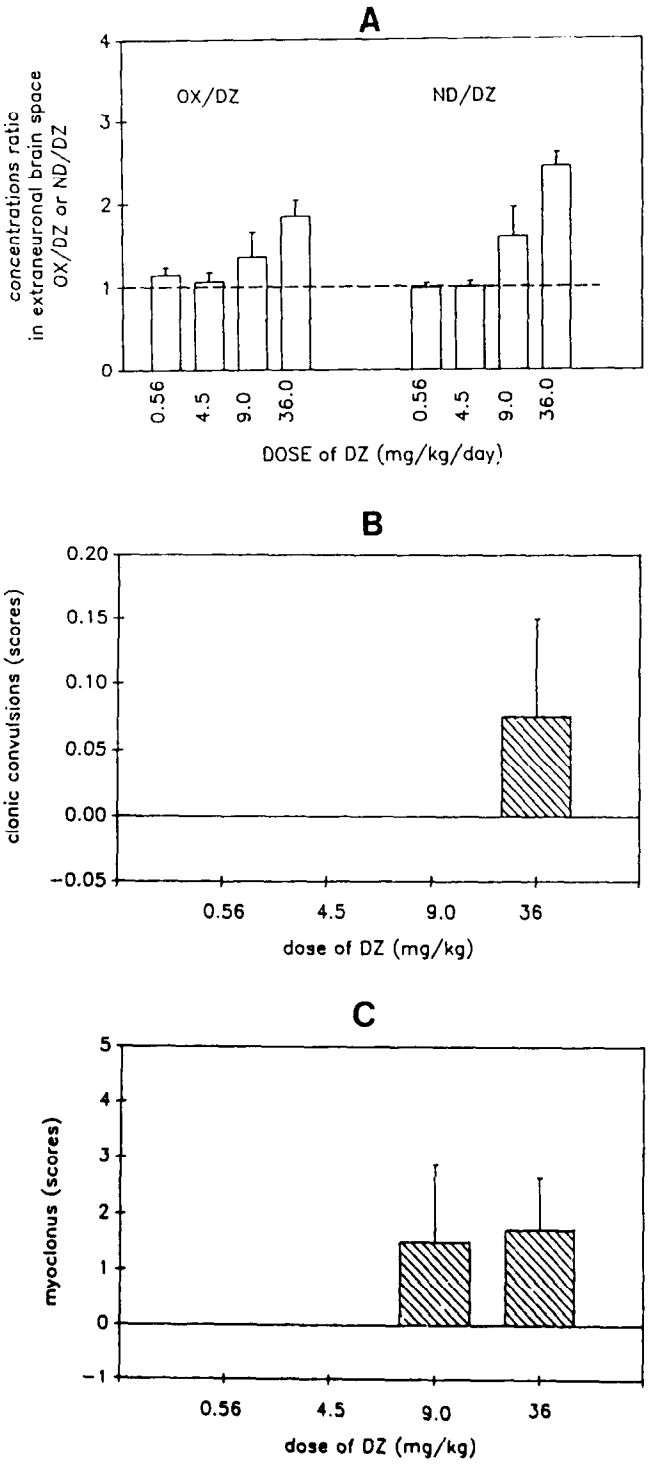

FIG. 4. (A) Concentrations ratios (ND/DZ and OX/DZ) of free DZ and its metabolites, ND and OX, in extraneuronal brain space (intracranial microdialysis perfusate) in dogs physically dependent on different chronic doses of DZ. Relationships between flumazenil (18 mg/kg, orally) evoking clonic convulsions (B) and myoclonus (C) and the chronic dose of DZ are shown. Scores were determined as area under the time action curves (with subtracted placebo). The mean scores (± SEM) for each group of dogs dependent on different doses of DZ are presented.

tion of CSF or extraneuronal levels from plasma samples can bear a significant error.

The observations that free levels of DZ in CSF did not increase with the dose of DZ and that there was a poor correlation between levels of free DZ in brain and plasma perfusates as well as between CSF and plasma appear to be inconsistent with the findings that in vitro, plasma protein binding of DZ does not change significantly with concentration and is not affected by the interactions between DZ and its metabolite(s) (3,33,41), and in vitro (the present study), the free fractions of DZ do not significantly differ among groups of dogs dependent on different doses of DZ. As reported here, the extent of binding of DZ varies greatly between dogs either within or between different doses of DZ, which is also the case in humans (1,14,22). Thus, it appears that the small between-dose difference in the levels of DZ together with the between-subject variability in the extent of protein binding result in a lack of significant regression of free DZ with dose. Several sources of variance in DZ levels have been indicated (15).

It is noteworthy that in dogs dependent on low doses of DZ, the steady-state levels of DZ and its metabolites were approximately equal, whereas with increasing doses of DZ, the contribution of unchanged DZ to all benzodiazepines present in plasma or brain (DZ and metabolites, taken as a total) decreased, which suggests altered metabolism of DZ. Conflicting reports employing different species, doses, routes, and frequencies of administration of DZ suggest either inhibition (25) or induction (36) of demethylation of DZ, inhibition of hydroxylation of ND (24,39,42), or lack of effect of ND on the kinetics of DZ (2). The data presented here show that under normalized conditions an extensive accumulation of ND and parallel accumulation of OX is related to the chronic administration of high doses of DZ.

The degree of dependence is thought to be related to receptor occupancy. A good correlation has been reported between free plasma levels, receptor occupancy, and the anticonvulsant effect of flunitrazepam (21), and between the total brain levels and ex vivo receptor occupancy for acutely administered clonazepam, lorazepam (32), and OX and DZ (18). It must be emphasized that either the benzodiazepines employed in these studies were not metabolized to pharmacologically active compounds or the receptor occupancy was estimated for the parent drug and metabolites taken as a total. The present data indicate that dogs physically dependent on increasing doses of DZ have progressively more free ND and OX and less free DZ in the extraneuronal brain space. Nordiazepam, which is thought to act as a partial agonist on central benzodiazepine receptors, has a lower intrinsic activity than DZ (13); acute tolerance does not develop to its anticonvulsant effect (12) and differs from DZ in its dependence-producing properties (30,31,38). Martin (28) suggested that DZ, ND, and OX act through different receptor subtypes producing different types of dependence characterized by qualitatively different signs of abstinence. This concept has been recently supported by the results of behavioral tests (9) and the data on tolerance and cross-tolerance to the anticonvulsant effect of benzodiazepines (34,35). As reported here, ND/DZ and OX/DZ concentration ratios in the extraneuronal brain space, and thus, the active components of the drug that are available for interaction with receptor(s) change with the dose of DZ. This observation suggests that the different signs of abstinence observed at the different levels of dependence on DZ are due to the differences in occupancies that are required to mediate various effects of DZ. In this regard, scores for several signs of abstinence are linearly related to free levels of ND at each level of

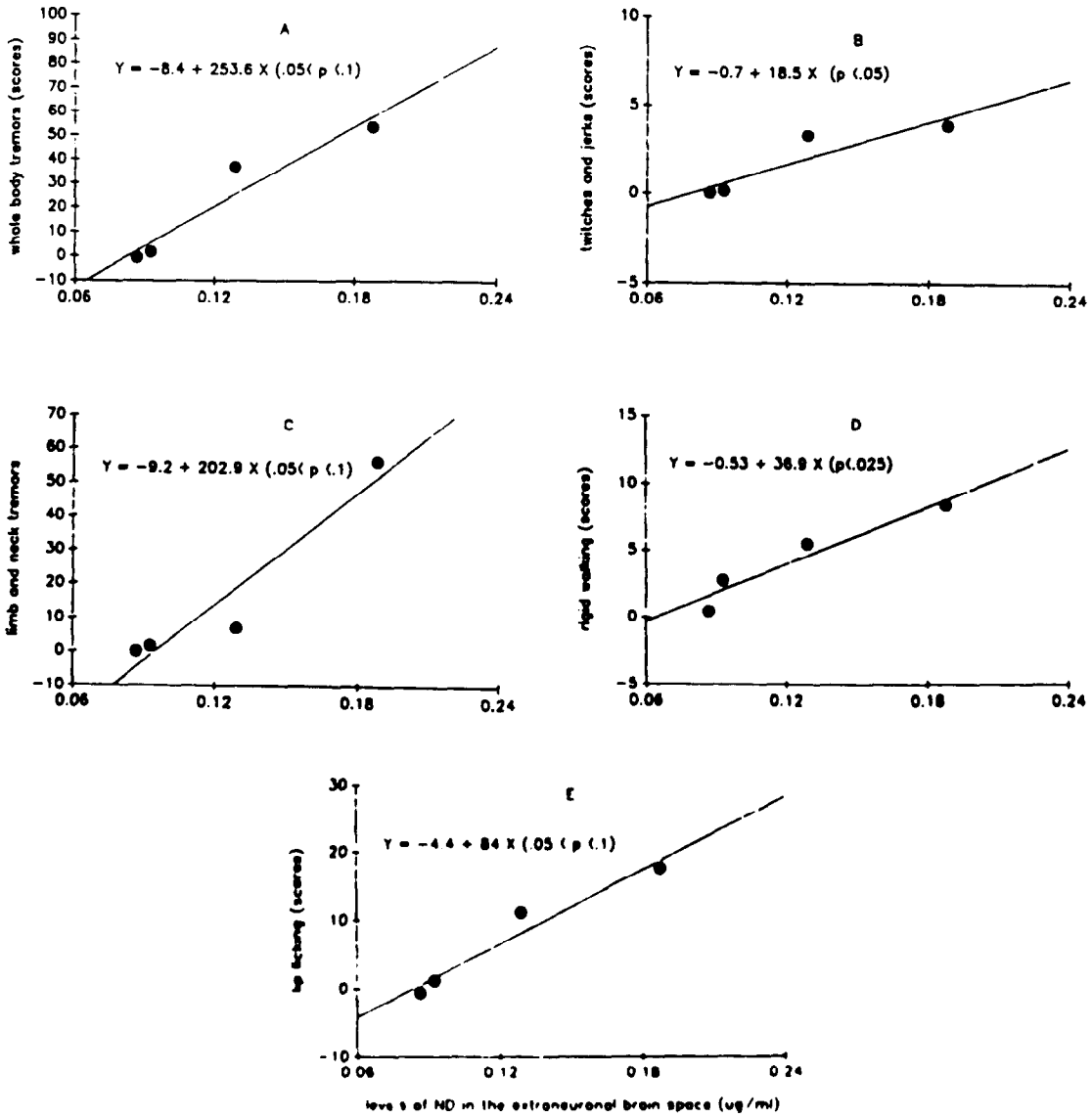

FIG. 5. Relationships between several signs of abstinence: whole body tremors (A), twitches and jerks (B), limb and neck tremors (C), rigid walking (D), and lip licking (E), and free levels of ND in intracranial perfusate. Scores were determined from areas under the time action curves (less placebo). Data are presented as mean scores vs. mean free levels for dogs dependent on each chronic dose of DZ.

dependence, whereas convulsive phenomena are evocable only when the levels of free metabolites exceed the levels of free DZ. These results indicate that knowledge of levels at the receptor site and the signs of abstinence may help elucidate the role of metabolites in physical dependence on DZ.

Although the metabolism of DZ in dogs and humans is similar, there are differences in clearance and accumulation of the parent drug and its metabolites when DZ is acutely administered (8). The present data indicate that in dogs physically dependent on doses of DZ equivalent to chronic therapeutic doses administered to humans, plasma levels of DZ and its metabolites are similar in the two species [cf., (37)]. Dogs physically dependent on higher doses of DZ accumulate ND and OX to a greater extent than DZ, which is also observed in drug abusers ingesting high doses of DZ (10,20). The results from this animal study may extended to humans with caution.

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